PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

58

PCT/EP 02/14895
10/500118

EPO - DG 1

27. 01. 2003

Intyg
Certificate

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

(71) *Sökande* / *Applicant (s)* — *Cell Therapeutics Scandinavia AB, Göteborg SE*

(21) *Patentansökningsnummer* / *Patent application number* — *0104471-8*

(86) *Ingivningsdatum* / *Date of filing* — *2001-12-28*

Stockholm, 2003-01-13

För Patent- och registreringsverket
For the Patent- and Registration Office

Christina Vängborg

Avgift
Fee 170:-

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

New Method

Field of the invention

The present invention concerns a method for the establishment of pluripotent human embryonic stem cells, the stem cells, differentiation of these cells into differentiated cells, the differentiated cells and the use of these differentiated cells in the preparation of medicaments. The undifferentiated pluripotent stem cells can be made to differentiate to a number of specialized cell types which can be utilized in the manufacture of medicaments for treating a number of pathologies of the pancreas, for example diabetes, or pathologies of the CNS, such as stroke, physical trauma, or Alzheimer's or Parkinson's diseases.

Background of the invention

A stem cell is a cell type that has a unique capacity to renew itself and to give rise to specialized or differentiated cells. Although most cells of the body, such as heart cells or skin cells, are committed to conduct a specific function, a stem cell is uncommitted, until it receives a signal to develop into a specialized cell type. What makes the stem cells unique is their proliferative capacity, combined with their ability to become specialized. For years, researchers have focused on finding ways to use stem cells to replace cells and tissues that are damaged or diseased. So far, most research has focused on two types of stem cells, embryonic and somatic stem cells. Embryonic stem cells are derived from the preimplantation embryo, i.e. blastocyst, whereas the somatic stem cells are present in the adult organism, e.g. within the bone marrow, epidermis and intestine. Pluripotency tests have shown that whereas the embryonic stem cells can give rise to all cells in the organism, including the germ cells, somatic stem cells have a more limited repertoire in descendent cell types. .

In 1998, investigators were for the first time able to isolate embryonic stem cells from human embryos (from now on termed human embryonic stem (hES) cells) and to grow them in culture. Presently, it is possible to keep at least some of these cell lines in a stable state for prolonged time, this is disclosed in e.g. US 5 843 780 and in US 6 200 806.

The procedure used in the patent specifications mentioned above depends on the use of blastocysts with an intact zona pellucida. Furthermore, the method disclosed in these patents specifically use inner cell mass cells that have been isolated by immunosurgery for plating on mouse embryonic feeder cells. This method has several drawbacks, for example, it is time consuming, technically difficult and results in low yields of stem cells. Taken together, these drawbacks make it a costly method.

Further, the present invention allows a successful derivation of human embryonic stem cell lines from hatched blastocysts and allows for derivation of hES cell lines after plating blastocysts with an intact trophectoderm onto feeder cells.

One of the difficulties with previously described methods has been to achieve an efficient attachment of the blastocysts to the feeder cells. This has resulted in low yields of end-product cells.

Perhaps the most far-reaching potential application of pluripotent human embryonic stem cells (hES) cells is the generation of cells and tissue that could be used for so-called cell therapies. Many diseases and disorders result from disruption of cellular function or destruction of tissues of the body. Today, donated organs and tissues are often used to replace ailing or destroyed tissue. Unfortunately, the number of people suffering from disorders suitable for treatment by these methods far outstrips the number of organs available for transplantation. The availability of hES cells and the intense research on developing efficient methods for guiding these cells towards different cell fates, e.g. insulin-producing β-cells, cardiomyocytes, and

dopamine-producing neurons, holds growing promise for future applications in cell-based treatment of degenerative diseases, such as diabetes, myocardial infarction and Parkinson's.

For patients suffering from type I diabetes the only cure that exists today is transplantation of intact pancreatic islets from diseased donors. The supply of such donated material is of course insufficient for treating all patients suffering from this disease. Therefore, derivation of pancreatic insulin-producing islet-like structures from hES cells, or for that matter from any stem cell, may be an attractive way of treating this disease in the future.

It has been shown that mouse ES cells can be made to differentiate to insulin producing cells (Lumelsky et al., 2001). In addition to the insulin producing β cells, these cells give rise to significant numbers of α cells and D cells. These cells, being mouse cells, are not applicable for the use in treating humans.

A requisite for the above-mentioned transplantation of cells is that the cells are of human origin. A further requisite for use in medicine is that the cells produced are free from any kind of infectious agents that may originate from serum or feeder cells of other origin than human. Without ensuring a procedure for isolating human embryonic stem cells without any infectious agents or other hazardous agents, the cells will not be approved for clinical applications.

Very recently, it was shown that it might be possible to differentiate human embryonic stem cells into insulin producing cells (Assady et al., 2001). However, the method according to these experiments gives low yields of insulin-positive cells. Further, it is technically and practically difficult, if not impossible, with this method to isolate enough insulin-positive cells for transplantation.

Summary of the invention

The inventors have now established novel methods for establishing human embryonic cell lines, growing them in an undifferentiated state, as well as for differentiating these cells into insulin-expressing pancreatic islet-like structures and neuron-like cells.

The inventors use a procedure for establishing hES cell lines, which alleviates the need for immunosurgery, and have devised a method to increase the efficiency of attachment of blastocysts to the feeder cells and growth of inner cell mass cells. By comparing the success-rate in using immunosurgery versus leaving the trophectoderm intact it is clear that the much simpler, faster and non-traumatic procedure of avoiding immunosurgery is more efficient than immunosurgery. Taken together, these novel procedures make the preparation of stem cell lines, and the differentiation of these cell lines commercially feasible.

The inventors have also developed a method for differentiating human stem cell lines derived by procedures disclosed herein into insulin-producing cells. The method gives unexpected high yields of insulin producing cells and this makes it for the first time possible to produce these cells in amounts great enough to make the method commercially useful. The inventors have also shown that the method can be applied for the production of differentiated neurons (neuron specific betaIII-tubulin positive). These cells have a potential use in the production of dopamine-producing neurons, which cells could be of use in the treatment of pathologies in the nervous system such as multiple schlerosis, spinal chord injury, encephalopathies, Parkinson's disease, Huntingdon's disease, stroke, traumatic brain injuries, hypoxia induced brain injuries, ischemia induced brain injuries, hypoglycemic brain injuries, degenerative disorders of the nervous system, brain tumors, or neuropathies in the peripheral nervous system.

According to the above, the invention is an essentially pure preparation of pluripotent human embryonic stem cells, which i) exhibits proliferation capacity in an undifferentiated state for more than 9 months when grown on mitotically inactivated embryonic feeder cells; ii) exhibits normal euploid chromosomal karyotype; iii) maintains potential to develop into derivatives of all types of germ layers; iv) exhibits at least two of the following molecular markers OCT-4, alkaline phosphatase, the carbohydrate epitopes SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, and the protein core of a keratin sulfate/chondroitin sulfate pericellular matrix proteinglycan recognized by the monoclonal antibody GCTM-2exhibits molecular markers OCT-4, alkaline phosphatase and GCTM-2, SSEA-4, v) does not exhibit molecular marker SSEA-1; vi) is capable of being made to differentiate into an essentially pure insulin producing cell line, and is also capable of forming islet-like structures.

The invention is also a method for isolating a pluripotent human embryonic stem cell line, comprising the steps of;

a) isolating a human blastocyst;
b) plating the blastocyst on mammalian embryonic fibroblast feeder cells;
c) culturing the blastocyst until colonies have formed that are large enough for manual passaging;
d) dissecting inner cell mass cells from other cell types produced in step c) followed by expanding them by growth on new embryonic feeder cells.

The invention is also a kit for performing the method according to the above, comprising at least two of the following components in separate compartments; hyaluronic acid, pronase, ES-cell medium, and human or mouse embryonic feeder cells

The invention is also a cell line established by the method above .

Further, the invention is a method for producing an essentially pure preparation of insulin-producing differentiated stem cells, comprising the steps of;

a) expanding human embryonic stem cells by growing these on an inactivated feeder cell layer in a suitable medium;

b) generating embryonic bodies by dissociating colonies formed in step a) into smaller aggregates or individual cells, followed by transferring said aggregates or individual cells to non-adherent containers where they are incubated in a suitable medium;

c) plating the embryonic bodies in containers in a suitable medium;

d) selecting nestin-positive neural precursors in ITFSn medium;

e) expanding pancreatic endocrine progenitor cells in N2 medium comprising B27 media complement and basic fibroblast growth factor;

f) withdrawing the basic fibroblast growth factor from the medium in step e).

The invention is also a kit for performing the method above, comprising at least two of the following components in separate compartments; mitomycin C, hES medium, EB-medium, ITSFn-medium, N2-medium, B27-media supplement, nicotinamide, and bFGF.

The invention is also an essentially pure preparation of differentiated stem cells, wherein the cells display the expression of pancreatic cell type markers, including at least one of insulin, Glut-2, Pdx-1, neurogenin, Pax4, Pax6, glucokinase, glucagon and somatostatin.

The invention is also an essentially pure preparation of differentiated stem cells, wherein the cells display the expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

The invention is also a use of a preparation according to the above for the manufacture of a medicament for the prevention or treatment of pathologies or diseases in the pancreas.

The invention is also a use of a preparation according to the above for the manufacture of a medicament for the treatment of pathologies or diseases in the nervous system.

The therapeutic potential for these cells is enormous. The method for producing these cells can be used for producing basically unlimited amounts of differentiated cells, and the cells themselves can be used for therapeutic purposes, for example in the field of diabetes, Parkinson's disease or any of the other diseases mentioned above.

The inventors show that nestin-producing neural progenitors derived from a human ES cell line can give rise to enriched populations of insulin-secreting β–cells. Furthermore, the hormone-producing cells spontaneously assemble into three-dimensional islet-like clusters. These results are important contributions towards the future goal to derive pancreatic islet-like clusters from human ES cells that can be used to substitute for the lack of functional pancreatic islets in diabetes.

The enrichment of insulin-expressing cells as well as their organization into pancreatic islet-like structures is essential for their future use in replacement therapy. A further advantage of the invention is the fact that the efficiency of insulin production is 10-20-fold higher than what has previously been reported.

Based on proof-of-principle experiments performed on mouse ES cells it is believed that ES cells may have the potential to be an efficient source of differentiated cell types that after transplantation can replace injured or diseased cells in various pathological conditions, such as diabetes, heart and neurodegenerative diseases and

spinal cord injuries. The inventors demonstration that a high number of insulin-producing β-cells can be derived from nestin-positive neural precursor cells that originate from human ES cells, clearly shows that the first hurdle, i.e. to obtain an enriched population of pancreatic islet cells, has been overcome.

The inventors can demonstrate the presence of insulin mRNA and protein at the cellular level, as well as displaying other cell-specific markers chosen from the group of; glucose transporter type 2, (Glut-2); glucokinase; neurogenin3 (ngn3); homeodomain transcription factor Pdx-1 (IPF1/IDX1/STF1); Pax4, which is a member of the paired homeodomain transcription factor; Pax6, which is a member of the paired homeodomain transcription factor family. Furthermore, the cells secrete surprisingly high amounts of insulin into the surrounding medium, suggesting that they are able to perform their normal function, i.e. to respond to elevated glucose by secreting insulin.

It should be noted that the procedure is not limited to producing pancreatic β-cells, but could be applied to any stem cell that can be derived from nestin-producing cells, such as the cells in the central nervous system. The argument for this is that the mechanisms that operate to control the development of both the central nervous system (CNS) and the pancreas are very similar.

Short description of the figures

Figure 1 demonstrates the differentiation of insulin-producing islet-like clusters. Scale bars: (A; B) 200 μM; (C, D, G, H) 20 μM; (E, F) 80 μM; (I) 5 μM.

Figure 1A) Phase-contrast image of undifferentiated human embryonic stem cells grown on mouse embryonic fibroblasts.

Figure 1B) Phase-contrast image of islet-like cell clusters obtained after the final differentiation phase.

Figure 1C) Confocal image of immunostainings of plated embryonic bodies after the selection stage for nestin.

Figure 1D) Confocal image of immunostainings of plated embryonic bodies after the selection stage for insulin.

Figure 1E) Confocal image of immunostainings of differentiated islet-like clusters for β-tubulin isotype III.

Figure 1F) Confocal image of immunostainings of differentiated islet-like clusters for insulin.

Figure 1G) Confocal image of immunostainings of differentiated islet-like clusters for β-tubulin isotype III.

Figure 1H) Confocal image of immunostainings of differentiated islet-like clusters for insulin.

Figure 1I) Confocal image of immunostainings of differentiated islet-like clusters for somatostatin.

Figure 2. Differentiation results in expression of pancreatic cell type markers. Total RNA extracted from undifferentiated hES cells (udES) and differentiated cells (dES) was analyzed for the presence of mRNAs for β-actin, OCT-4, insulin (Ins), GLUT-2 and glucagon (Gluc) by RT-PCR.

Figure 3 Differentiated hES cells secrete insulin into the medium. The amount of insulin secreted into the medium of differentiated (dES) hES cells was measured and compared to the amount of insulin in medium that has not been in contact with cells.

Figure 4A) Morphology of undifferentiated hES cell line SA01.

Figure 4B) Alkaline phosphatase in undifferentiated hES cell line SA01.

Figure 4C) Immunostaining of undifferentiated hES cell line SA01 with GCTM-2.

Figure 4D) Total RNA extracted from undifferentiated hES cell line SA01 was analyzed for the presence of OCT-4 mRNA by RT-PCR.

Figure 5A) Morphology of undifferentiated hES cell line SA02.

Figure 5B) Alkaline phosphatase in undifferentiated hES cell line SA02.

Figure 5C) Immunostaining of undifferentiated hES cell line SA02 with GCTM-2.

Figure 5D) Total RNA extracted from undifferentiated hES cell line SA02 was analyzed for the presence of OCT-4 mRNA by RT-PCR.

Figure 6A) Morphology of undifferentiated hES cell line SA03.

Figure 6B) Alkaline phosphatase in undifferentiated hES cell line SA03.

Figure 6C) Immunostaining of undifferentiated hES cell line SA01 with GCTM-3..

Figure 6D) Total RNA extracted from undifferentiated hES cell line SA03 was analyzed for the presence of OCT-4 mRNA by RT-PCR.

Definitions

As used herein, the term "embryonic stem cell" is denoted ES cells, and the human form is termed hES cells.

As used herein, the term "embryoid body " is denoted EB.

As used herein, the term "EF cells" means embryonic fibroblast feeder. These cells could be derived from any mammal, such as mouse or human.

One suitable medium used in the invention is termed "ES-cell medium" or "ES-medium" and may be comprised of; KNOCKOUT® Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 µg/ml streptomycin, , 0,1 mM non-essential amino acids, 2mM L-glutamine, 100µM β-mercaptoethanol, 4ng/ml human recombinant bFGF (basic fibroblast growth factor), and 2000 U/ml LIF (leukemia inhibitory factor) (Gibco BRL).

Another suitable medium for the present invention is "EB-medium" or "embryonic body-medium", this may be comprised as follows; KNOCKOUT® Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 µg/ml streptomycin, 0,1 mM non-essential amino acids, 2mM L-glutamine and 100µM β-mercaptoethanol (Itskovitz-Eldor, J. et al., 2000).

Another medium for the present invention is "ITSFn medium" which may be comprised of; DMEM/F12 supplemented with 2 mM L-glutamine (GibcoBRL), 5 µg/ml insulin, 50 µg/ml human apotransferrin, 5,2 ng/ml sodium selenite, 5 µg/ml fibronectin (Sigma) (Rizzino and Crowley, 1980).

Another medium for the present invention is N2which may be comprised of; DMEM/F12, N2 and B27 supplements, 2 mM L-glutamine, (GibcoBRL), 5 mM glucose, 1,2 g/l sodium bicarbonate, 25 µg/ml insulin, 100 µg/ml human apotransferrin;, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite, 10 ng/ml bFGF (Sigma) (Lee et al., 2000; Johe et al., 1996; Lumelsky et al., 2001)

The term "essentially pure" with regards to an insulin producing cell line, as defined herein, is meant to define a cell population in which the proportion of insulin producing cells is at least 25% of the total number of cells, preferably at least 35% of the total number of cells, or more preferably at least 45% of the total number of cells and/or wherein said insulin producing cells produce at least 300ng insulin/mg total protein, or preferably at least 380ng insulin/mg total protein, or most preferably at least 450ng insulin/mg total protein.

The term "essentially pure" with regards to a neuronal cell line, as defined herein, is meant to define a cell population in which the proportion of neuronal cell marker-carrying cells, is at least 20% of the total number of cells, preferably at least 35% of the total number of cells, or more preferably at least 40% of the total number of cells.

The insulin producing cells can be further defined by the presence of at least one of the following cell markers; glucose transporter type 2, (Glut-2); glucokinase; neurogenin3 (ngn3); homeodomain transcription factor Pdx-1 (IPF1/IDX1/STF1); Pax4, which is a member of the paired homeodomain transcription factor; Pax6, which is a member of the paired homeodomain transcription factor family. Cells expressing glucagon and somatostatin are also derived by the present differentiation method.

The undifferentiated hES according to the present invention are defined by the following criteria; they were isolated from human pre-implantation embryos, i.e. blastocysts, and exhibit an unlimited proliferation capacity in an undifferentiated state when grown on mitotically inactivated EFs; they exhibit a normal chromosomal karyotype; they express typical markers for undifferentiated hES, e.g. OCT-4, alkaline phosphatase, the carbohydrate epitopes SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, and the protein core of a keratin sulfate/chondroitin sulfate pericellular matrix proteinglycan recognized by the monoclonal antibody GCTM-2, and do not show any expression of the carbohydrate epitope SSEA-1. Furthermore, pluripotency tests in vitro (EBs) and in vivo (teratomas) demonstrate differentiation into derivatives of all germ layers. More importantly, these cells are capable of being made to differentiate into an essentially pure insulin producing cell line, and are also capable of forming islet-like structures.

Detailed description of the invention

Human blastocysts may be derived from frozen or fresh human in vitro fertilized embryos. Human embryonic stem cell lines may be isolated either from spontaneously hatched blastocysts or from expanded blastocysts with an intact zona pellucida.

For hatched blastocysts the trophectoderm may be left intact. Either hatched blastocysts or blastocysts with an intact zona pellucida may be put on inactivated feeder cells. Feeder cells can be of mouse or human origin.

A suitable medium for plating the blastocysts onto feeder cells can be ES-medium that may be complemented with hyaluronic acid, which seems to promote the attachment of the blastocysts on the feeder cells and growth of the inner cell mass. Hyaluronan (HA) is an important glycosaminoglycan constituent of the extracellular matrix in joints. It appears to exert its biological effects through binding interactions with at least two cell surface receptors: CD44 and receptor for HA-

mediated motility (RHAMM), and to proteins in the extracellular matrix. The positive effects of HA during the establishment of hES cells may be exerted through its interactions with the surfactant polar heads of phospholipids in the cell membrane, to thereby stabilize the surfactant layer and thus lower the surface tension of the inner cell mass or blastocyst which may result in increased efficiency in binding to the EF cells. Alternatively, HA may bind to its receptors on the inner cell mass or blastocyst and/or to the EF cells and exert biological effects which positively influence the attachment and growth of the inner cell mass. According to this, other agents that may alter the surface tension of fluids, or in other ways influence the interaction between the blastocyst and feeder cells can also be used in instead of hyaluronic acid. A brief pronase (Sigma) treatment of blastocysts with an intact zona pellucida results in the removal of the zona. Other types of proteases with the same or similar protease activity as pronase may also be used. The blastocysts can be plated onto said inactivated feeder cells following the pronase treatment.

After being plated onto feeder cells, their growth is monitored and when the colony is large enough for manual passaging (approximately 1-2 weeks after plating), the cells may be dissected from other cell types and expanded by growth on new feeder cells.

It is possible to select for nestin-producing neural precursor cells by plating human embryoid bodies (EBs) on tissue culture plastics under serum-free conditions. This procedure promotes survival of nestin-positive cells, whereas most other cell types die. At this stage a few insulin-positive cells may be detected among the nestin-producing progenitors (Fig. 1C,D). These cells may then be expanded in the presence of a mitogen, e. g. basic fibroblast growth factor (bFGF) (Lee et al., 2000; Brewer et al., 1993), followed by mitogen withdrawal to promote cell differentiation. In addition, nicotinamide can be added at a final concentration of 10 mM to promote β cell differentiation (Otonkoski et al., 1993). In our experiments, cells became arranged in three-dimensional clusters surrounded by neuronal-like cells dur-

ing the differentiation stage (Fig. 1B). Analysis of the expression of various pancreatic endocrine and neuronal markers, demonstrated that the number of insulin-positive cells as well maturing neurons expressing neuron-specific β-III tubulin (TUJ1) increased during the differentiation stage. The cell-clusters were highly enriched for insulin-positive cells and surrounded by TUJ1-expressing cells (Fig. 1E-H). Whereas few somatostatin-producing D cells and no glucagon-producing alpha cells (α cells) were observed (Fig. 1I and data not shown), mRNAs for glucagon and somatostatin were detected (Fig. 2 and data not shown). This is in contrast to mouse ES cells, which, in addition to β cells, give rise to significant numbers of α cells and D cells.

Whereas mRNAs for typical markers of undifferentiated ES cells, such as OCT-4 and alkaline phosphatase, were selectively detected in undifferentiated ES cells (Figs. 2, 4B, 4D, 5B, 5D, 6B, 6D), mRNAs for pancreatic cell type markers, including insulin, Pdx-1, Glut-2, glucokinase, neurogenin-3, Pax4, Pax6, glucagon and somatostatin (Fig. 2 and data not shown), were selectively observed in cells derived from the selection/differentiation scheme.

To evaluate the efficiency of β-cell differentiation, the percentage of insulin-positive cells within the median plane of the cell clusters can be estimated using confocal microscopy. In our experiments, approximately 47% of the cells expressed insulin. Importantly, this efficiency is 10-20-fold higher than previously reported. Further analysis of the insulin-positive clusters revealed a topological variation in both the number of insulin-expressing cells and the amount of insulin expressed, i.e. insulin-expressing cells were enriched within the center of the clusters and cells distributed within the center of the clusters expressed higher levels compared to cells in the periphery (Fig. 1E-H).

To test if the insulin-producing cells may represent β-cells, one experiment may be to examine whether the cells are capable of secreting insulin into the medium. Insulin is removed from the differentiation medium and insulin is measured in

the medium after a short (e.g. two days) incubation at the final phase of the differentiation period. Detection of high levels of insulin in the medium, would indicate that the insulin-expressing cells represent mature β-cells. In our experiments, this is indeed the case (Fig. 3).

In addition to the therapeutic potentials, the presented human ES cell system offers an attractive in vitro system for studying the molecular basis for the differentiation of human insulin-producing β-cells and their assembly into functional insulin-producing islet-like clusters.

In accordance to the above, it is one object of the present invention to provide a method for establishing a stable undifferentiated embryonic stem cell line. As a starting material for this procedure, blastocysts are used. It is a well-known procedure to prepare the blastocysts and a person skilled in the art will be able to perform the preparation of blastocysts. Procedures for this may be found in Gardner et al, Embryo culture systems, In Trounson, A. O., and Gardner, D. K. (eds), *Handbook of in vitro fertilization, second edition.* CRC Press, Boca Raton, pp. 205-264; Gardner et al, *Fertil Steril*, 74, **Suppl 3**, O-086; Gardner et al, *Hum Reprod*, **13**, 3434,3440; Gardner et al, *J Reprod Immunol*, In press; and Hooper et al, *Biol Reprod*, 62, **Suppl 1**, 249.

It is a further object of the present invention to provide a pluripotent human embryonic stem cell line that is capable to differentiate into an essentially pure insulin producing cell line.

It is a further object of the invention to provide a pluripotent human embryonic stem cell line that is capable to differentiate into an essentially pure insulin producing cell line, which forms an islet like structure characterized by neuronal cells and insulin producing β-cells that are enriched within the center.

It is another object of the present invention to provide a pluripotent human embryonic stem cell line that is capable to differentiate into an essentially pure insulin producing cell line, whereby said insulin producing cell line is characterized by the proportion of insulin producing cells being at least 40% of the total number of cells, preferably at least 45% of the total number of cells, or more preferably at least 50% of the total number of cells and/or wherein said insulin producing cells produce at least 300 ng insulin/mg total protein, or preferably at least 380 ng insulin/mg total protein, or most preferably at least 450 ng insulin/mg total protein,

Yet a further object of the invention is to provide a method for producing an essentially pure preparation of differentiated, insulin secreting β-cells from human stem cells, comprising the steps of;

a) propagating human embryonic stem cells by growing cells on an embryonic feeder cell layer in a suitable medium such as human embryonic stem cell medium as defined herein,

b) generating embryonic bodies by enzymatic treatment or by manual dissection, for example by cutting with glass capillaries, whereby colonies are dissociated into individual cells or smaller aggregates with an approximate size of (0.2-0.5)x(0.2-0.5) mm, followed by transferring said pieces to containers of a non-adherent material,

c) plating the embryonic bodies on tissue culture containers in embryonic body medium,

d) selecting nestin-positive neural precursors in a suitable medium such as ITSFn medium,

e) expanding pancreatic endocrine progenitor cells in N2-medium comprising B27 media supplement and basic fibroblast growth factor,

f) withdrawing basic fibroblast growth factor from the medium.

The feeder cells can be derived from any mammal; feeder cells from human or mouse species are preferred.

The manual dissection in step b) may be performed by using glass capillaries as a cutting tool.

It is a further object of the invention to provide an essentially pure preparation of differentiated cells obtainable by the method according to the differentiation method above, in which the cells are characterized by the expression of pancreatic cell type markers, including at least one of insulin, Glut-2, Pdx-1, neurogenin, Pax4, Pax6, glucokinase, glucagon and somatostatin, whereby the amount of said insulin produced is at least 450 ng insulin/mg total protein,

Yet another object of the invention is to provide an essentially pure preparation of differentiated cells, obtainable by the method according to claim 1, which cells are characterized by the expression of pancreatic cell type markers, including insulin, Glut-2, Pdx-1, neurogenin, Pax4, Pax6, glucokinase, glucagon and somatostatin, in which the proportion of insulin-producing cells is more than 50% of the total number of cells.

Yet another object of the invention is to provide an essentially pure preparation of differentiated stem cells obtainable by the method according to the differentiation method described above, characterized by the expression of CNS cell type markers, including at least one of neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

Procedures for the detection of cell markers can be found in Gage, F. H., Science, 287:1433-1438 (2000). The procedures are procedures that are well known for the skilled person and include methods such as RT-PCR or immunological assays where antibodies directed against the cell markers are used.

A further object of the invention is to provide an essentially pure preparation of embryonic stem cells that can be made to differentiate into oligodendrocytes, and also to provide an essentially pure preparation of oligodendrocytes prepared by this method. Oligodendrocytes can be characterized by the presence of cell markers such s RIP, GalC or O4.

According to the invention, cells that have been differentiated into neuronal type cells, carrying markers such as those mentioned above, or into oligodendrocytes, may be used in the preparation of a medicament for the treatment and/or prevention of diseases such as multiple schlerosis, spinal chord injury, encephalopathies, Parkinson's disease, Huntingdon's disease, stroke, traumatic brain injuries, hypoxia induced brain injuries, ischemia induced brain injuries, hypoglycemic brain injuries, degenerative disorders of the nervous system, brain tumors, or neuropathies in the peripheral nervous system.

A further object of the invention is to provide cells that may be used for the preparation of a medicament for treating and/or preventing diseases that may be cured by "cell genesis". By the term "cell genesis" is meant the generation of new cells such as neurons, oligodendrocytes, schwann cells, astroglial cells, all blood cells, chondrocytes, cardiomyocytes, oligodendroglia, astroglia, and/or different types of epithelium, endothelium, liver-, kidney-, bone-, connective tissue-, lung tissue-, exocrine and endocrine gland tissue-cells.

Yet another object of the invention is to provide an essentially pure preparation of differentiated stem cells produced by the method described above, characterized by being comprised of insulin-producing cells in the center and neuron-type cells.

In one embodiment of the invention, there is provided the use of the preparation of differentiated cells mentioned above, for the manufacture of a medicament for the prevention and/or treatment of pathologies or diseases in the pancreas.

In one embodiment of the invention, there is provided the use of the preparation of the above mentioned differentiated cells, for the manufacture of a medicament for the prevention and/or treatment of diabetes.

In one embodiment of the invention, there is provided the use of the preparation of the above mentioned differentiated cells, for the prevention and/or treatment of type 1 diabetes.

In one embodiment of the invention, there is provided the use of the preparation of the above mentioned differentiated cells, for the manufacture of a medicament for the treatment of pathologies or diseases in the nervous system.

In one embodiment of the invention, there is provided the use of a preparation of the above mentioned differentiated cells, for the manufacture of a medicament for the treatment of pathologies or diseases in the nervous system, chosen from the group of multiple schlerosis, spinal chord injury, encephalopathies, Parkinson's disease, Huntingdon's disease, stroke, traumatic brain injuries, hypoxia induced brain injuries, ischemia induced brain injuries, hypoglycemic brain injuries, degenerative disorders of the nervous system, brain tumors, or neuropathies in the peripheral nervous system.

In one embodiment of the invention, a kit for performing the differentiation method mentioned above, comprising at least two of the following components in separate compartments; mitomycin C, ES medium, EB-medium, ITSFn-medium, N2-medium, B27-media supplement, nicotinamide, or bFGF is provided.

In yet another embodiment of the invention, a kit according to claim 10, further comprising a preparation of undifferentiated embryonic stem cells is provided. A preparation of undifferentiated stem cells prepared by the method according to the preparation method described above is preferred.

The invention will now be described with reference to the following examples. The examples are included herein for illustrative purposes only and are not intended to limit the scope of the invention in any way. The methods described herein are well known to a person skilled in the art and all reagents and buffers are readily available, either commercially or easily prepared according to well-established protocols in the hands of a person skilled in the art. All incubations were in 37°C, under a CO_2 atmosphere.

Example 1

Establishment of an essentially pure preparation of undifferentiated stem cells from spontaneously hatched blastocysts.

Human blastocysts were derived from frozen or fresh human in vitro fertilized embryos. Spontaneously hatched blastocysts were put directly on EF cells in ES cell medium (KNOCKOUT Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT Serum replacement, and the following constituents at the final concentrations: 50 units/ml penicillin, 50 µg/ml streptomycin, 0.1 mM non-essential amino acids, 2mM L-glutamine, 100mM β-mercaptoethanol, 4ng/ml human recombinant bFGF (basic fibroblast growth factor), and 2000 U/ml LIF (Gibco BRL)), supplemented with 0.125 mg/ml hyaluronic acid. After plating the blastocysts on the EF cells, growth was monitored and when the colony was large enough for manual passaging approximately 1-2 weeks after plating) the inner cell mass cells were dissected from other cell types and expanded by growth on new EF cells.

Example 2

Establishment of an essentially pure preparation of undifferentiated stem cells from blastocysts with an intact zona pellucida.

For blastocysts with an intact zona pellucida, a brief pronase (10 U/ml, Sigma) incubation in rS2 (ICM-2) medium (Vitrolife, Gothenburg, Sweden) was used to digest the zona, after which the blastocyst was put directly on the EF cell layer in ES medium supplemented with hyaluronic acid (0.125 mg/ml).

Example 3

The cells were harvested for RT-PCR and histological (alkaline phosphatase) and immunocytochemical analysis (see below).

RNA isolation and RT-PCR. Total cellular RNA was prepared using Rneasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The cDNA synthesis was carried out using AMV First Strand cDNA Synthesis Kit for RT-PCR (Roche) and PCR using Platinum Taq DNA Polymerase (Invitrogen). Histochemical staining for alkaline phosphatase was carried out using commercially available kit (Sigma) following the manufacturer's recommendations.

Example 4

Mouse embryonic fibroblasts feeder cells were cultivated on tissue culture dishes in EMFI-medium: DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 10% FCS (Fetal Calf Serum), 0,1mM β-mercaptoehanol (), 50 units/ml penicillin, 50 µg/ml streptomycin and 2mM L-glutamine (GibcoBRL). The feeder cells were mitotically inactivated with Mitomycin C (10µg/ml, 3 hrs). Human embryonic stem cell-colonies were expanded by manual dissection onto inactivated mouse embryonic fibroblasts feeder cells.

Human embryonic stem cells were cultured on mitotically inactivated mouse embryonic fibroblasts feeder cells in tissue culture dishes with ES-cell medium: KNOCKOUT® Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 µg/ml streptomycin, 0,1 mM non-essential amino acids, 2mM L-glutamine, 100µM β-mercaptoethanol, 4ng/ml human recombinant bFGF (basic fibroblast growth factor), and 2000 U/ml LIF (leukemia inhibitory factor) (Gibco BRL). Seven days after passage the colonies were large enough to generate embryoid bodies.

ES cell colonies were cut with glass capillaries into 0.4x0.4 mm pieces and plated on non-adherent bacterial culture dishes containing EB-medium: KNOCKOUT® Dulbecco's Modified Eagle's Medium, supplemented with 20% KNOCKOUT® Serum replacement and the following constituents at their respective final concentrations: 50 units/ml penicillin, 50 µg/ml streptomycin, 0,1 mM non-essential amino acids, 2mM L-glutamine and 100µM β-mercaptoethanol (Itskovitz-Eldor, J. et al., 2000). The EBs, including cystic EBs, formed over a 7-9-day period.

The embryoid bodies were transferred to tissue culture plates with EB medium. Plating of EBs normally took place during a 7-10-day period. After plating the medium was changed to ITSFn medium: DMEM/F12 supplemented with 2 mM L-glutamine (GibcoBRL), 5 µg/ml insulin, 50 µg/ml human apotransferrin, 5,2 ng/ml sodium selenite, 5 µg/ml fibronectin (Sigma) (Rizzino and Crowley, 1980). The selection phase within the ITSFn medium lasted for seven days.

This is a critical step for increasing the proportion of nestin-positive cells because many other cell types die in this serum-free medium. The ITSFn medium was changed every other day. Immunocytochemical analysis was performed at day 7 of the selection phase.

The cells were dissociated by trypsin (trypsin 0,05 %, EDTA 0,04 % in PBS) and plated on tissue culture dishes, precoated with 15 µg/ml poly-L-ornithine (Sigma) and 1 µg/ml laminin (GibcoBRL) at a concentration of 2×10^5 cells/cm^2. Cells were also plated on glass coverslips, precoated as above, for immunocytochemical analysis. An expansion medium (N2 medium) was used: DMEM/F12, N2 supplement, B27 supplement, 2 mM L-glutamine, (GibcoBRL), 5 mM glucose, 1,2 g/l sodium bicarbonate, 25 µg/ml insulin, 100 µg/ml human apotransferrin;, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite, 10 ng/ml bFGF (Sigma) (Lee et al., 2000; Johe et al., 1996; Lumelsky et al., 2001). The DMEM/F12 contained 17,5 mM glucose, therefore the total glucose concentration was 22,5 mM. The medium was changed every other day during a 7-day period. After two days, a morphological rearrangement was observed, the cells began to assemble into islet-like clusters. Between the clusters neuron-like projections could be observed.

To induce the differentiation of the progenitor cells to insulin secreting islet-like clusters a differentiation medium with the following composition was used: DMEM/F12, N2 and B27 supplements, 2 mM L-glutamine, (GibcoBRL), 5 mM glucose, 1,2 g/l sodium bicarbonate, 25 µg/ml insulin, 100 µg/ml human apotransferrin;, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite and 10 mM nicotinamide (Sigma) (Lee et al., 2000; Johe et al., 1996; Lumelsky et al., 2001). The medium was changed every other day during a 12-14 day period.

Example 5

The following experiment was carried out to measure the amount insulin secreted into the medium. The differentiation medium was exchanged for insulin-free medium consisting of DMEM/F12, N2 and B27 supplements, 10% FCS, 2 mM L-glutamine (GibcoBRL), 5 mM glucose and 10 mM nicotinamide (Sigma), for two days at the end of the differentiation phase. Insulin content in medium was meas-

ured using a human insulin radioimmunoassay (RIA) kit (Pharmacia insulin RIA 100, Pharmacia & Upjohn Diagnostics AB). Cells were solubilized and protein concentrations were determined using Bio-Rad protein assay system (Bio-Rad).

The cells were harvested for RT-PCR and immunocytochemical analysis and the medium collected for RIA at day 14 of the differentiation stage.

The amount of insulin produced by these cells is estimated by measuring the amount of insulin secreted into the media, and is then correlated to the amount of total protein in the cell.

Example 6

RNA isolation and RT-PCR. Total cellular RNA was prepared using Rneasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The cDNA synthesis was carried out using AMV First Strand cDNA Synthesis Kit for RT-PCR (Roche) and PCR using Platinum Taq DNA Polymerase (Invitrogen). Forward and reverse primer sequences from 5' to 3' direction and the length of the amplified products were as follows: OCT-4: GGCGTTCTCTTTGGAAAGGTGTTC and CTCGAACCACATCCTTCTCT (312 bp), β-actin: TGGCACCACACCTTCTACAATGAGC and GCACAGCTTCTCCTTAATGTCACGC (400 bp), GLUT-2: GTACAATGACAGAAGATAAG and TGCTACTAACATGGCTTTGA (398 bp), glucagon: GAATTCATTGCTTGGCTGGT and CATTTCAAACATCCCACGTG (255 bp) and insulin: GCCTTTGTGAACCAACACCTG and GTTGCAGTAGTTCTCCAGCTG (261 bp).

The results are seen in figures 1 and 2. The percentage of insulin producing cells varies from 22-62% (n=6), mean=47%.

Example 8

Immunocytochemical analysis: Cells grown on poly-ornithine/laminine–coated glass coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes. Immunofluorescence was carried out with the use of standard protocols. Briefly, the cells were washed after PFA fixation, permeabilised in 0,25% Triton-X-100 in PBS for 10 minutes, washed in PBS, blocked in PBS supplemented with 5% goat serum and 0,1% Triton-X-100, after which the primary antibodies were diluted in PBS supplemented with 1% goat serum, 0,1% Triton-X-100 and added for 3 hours at room temperature. The following primary antibodies were used at following dilutions: rabbit anti-glucagon 1:50 (Zymed), rabbit anti-somatostatin 1:50 (Zymed), guinea pig anti human insulin serum 1:500 (Linco), mouse anti nestin antibody 1:50 (BD Transduction Laboratories) and monoclonal anti-β-tubulin isotype III 1:50 (Sigma). After the primary antibody incubation the cells were washed in PBS supplemented with 0,1% Triton-X-100, followed by incubation with the conjugated secondary antibodies and streptavidin, each for 1 hour. For detection of primary antibodies the following secondary antibodies were used: Biotinylated anti guinea pig 1:1000 (Vector), FITC-conjugated streptavidin 1:500, Cy3 anti-mouse 1:300, Cy3 anti-rabbit 1:100, Cy3 conjugated streptavidin 1:1000 (Jackson).

REFERENCES

1. Itskovitz-Eldor, J. et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* **6**, 88-95. (2000).

2. Rizzino, A. & Crowley, C. Growth and differentiation of embryonal carcinoma cell line F9 in defined media. *Proc Natl Acad Sci U S A* **77**, 457-61. (1980).

3. Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. & McKay, R. D. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* **18**, 675-9. (2000).

4. Johe, K. K., Hazel, T. G., Muller, T., Dugich-Djordjevic, M. M. & McKay, R. D. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev* **10**, 3129-40. (1996).

5. Lumelsky, N. et al. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* **292**, 1389-94. (2001).

6. Brewer, G. J., Torricelli, J. R., Evege, E. K. & Price, P. J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* **35**, 567-76. (1993).

7. Otonkoski, T., Beattie, G. M., Mally, M. I., Ricordi, C. & Hayek, A. Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *J Clin Invest* **92**, 1459-66. (1993).

8. Assady, S., Maor, G., Michal, A., Itskovitz-Eldkor, J., Skkorecki, K.L. & Tzukerman, M., Insulin Production by human Embryonic Stem Cells. *Diabetes* **50**, 1691-1697, 2001.

9. Gardner et al, Embryo culture systems, In Trounson, A. O., and Gardner, D. K. (eds), *Handbook of in vitro fertilization, second edition*. CRC Press, Boca Raton, pp. 205-264;

10. Gardner et al, *Fertil Steril*, 74, **Suppl 3**, O-086; 11. Gardner et al, *Hum Reprod*, **13**, 3434,3440;

12. Gardner et al, *J Reprod Immunol*, In press;

13. Hooper et al, *Biol Reprod*, 62, **Suppl 1**, 249.

14. Gage, F. H., *Science*, **287**:1433-1438 (2000).

Claims

1) An essentially pure preparation of pluripotent human embryonic stem cells, which i) exhibits proliferation capacity in an undifferentiated state for more than 9 months when grown on mitotically inactivated embryonic feeder cells; ii) exhibits normal euploid chromosomal karyotype; iii) maintains potential to develop into derivatives of all types of germ layers; iv) exhibits at least two of the following molecular markers OCT-4, alkaline phosphatase, the carbohydrate epitopes SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, and the protein core of a keratin sulfate/chondroitin sulfate pericellular matrix proteinglycan recognized by the monoclonal antibody GCTM-2exhibits molecular markers Oct-4, alkaline phosphatase and GCTM-2, SSEA-4, v) does not exhibit molecular marker SSEA-1; vi) is capable of being made to differentiate into an essentially pure insulin producing cell line, and is also capable of forming islet-like structures.

2) The preparation according to claim 1wherein the proportion of insulin producing β-cells is higher than 40%, or preferably higher than 45%, or more preferably higher than 50%.

3) The preparation according to claim 1 or 2, which produces at least 300ng insulin/mg total protein, preferably at least 380ng insulin/mg total protein or most preferably at least 450ng insulin/mg total protein.

4) A method for isolating a pluripotent human embryonic stem cell line, comprising the steps of;
   a) isolating a human blastocyst;
   b) plating the blastocyst on mammalian embryonic fibroblast feeder cells;
   c) culturing the blastocyst until colonies have formed that are large enough for manual passaging;

d) dissecting inner cell mass cells from other cell types produced in step c) followed by expanding them by growth on new embryonic feeder cells.

5) The method according to claim 4 in which the blastocyst in step a) is a spontaneously hatched blastocyst.

6) The method according to claim 4 in which the blastocyst has been treated with pronase prior to step b).

7) The method according to claim 4-6 in which step b) is performed in the presence of hyaluronic acid.

8) The method according to claim 4-7 in which step c) is performed in the presence of hyaluronic acid.

9) The method according to claims 4-8 in which the feeder cells are of mouse or human origin.

10) A cell line established by the method according to claims 4-9.

11) Method for producing an essentially pure preparation of insulin-producing differentiated stem cells, comprising the steps of;

a) expanding human embryonic stem cells by growing these on an inactivated feeder cell layer in a suitable medium;

b) generating embryonic bodies by dissociating colonies formed in step a) into smaller aggregates or individual cells, followed by transferring said aggregates or individual cells to non-adherent containers where they are incubated in a suitable medium;

c) plating the embryonic bodies in containers in a suitable medium;

d) selecting nestin-positive neural precursors in ITFSn medium;

e) expanding pancreatic endocrine progenitor cells in, N2-medium comprising B27 media complement and basic fibroblast growth factor;

f) changing the medium to a basic fibroblast growth factor-free N2 medium.

12)Method according to claim 11 in which the human embryonic stem cells are the cells according to claims 1-3.

13)Method according to claims 11-12 in which the medium used in step a) is human embryonic stem cell medium.

14)Method according to claim 11-13 in which the medium used in step b) is embryonic body medium.

15)Method according to claims 11-14 in which the medium used in step c) is embryonic body medium.

16)Method according to claims 11-15 in which nicotinamide is added after step f).

17)An essentially pure preparation of differentiated stem cells, wherein the cells display the expression of pancreatic cell type markers, including at least one of insulin, Glut-2, Pdx-1, neurogenin, Pax4, Pax6, glucokinase, glucagon and somatostatin.

18)The preparation according to claim 16, which is capable of producing at least 320ng insulin/mg total protein, preferably at least 380ng insulin/mg total protein or most preferably at least 420ng insulin/mg total protein.

19)The preparation according to claims 16-17, in which preparation the proportion of insulin producing cells is at least 35%, preferably at least 45% or most preferably at least 50%.

20) The preparation according to claims 16-18, characterized its organization into islet-like structures comprising an inner core of β-cells surrounded by an outer layer of neuron-type cells, which neuron-type cells display expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

21) The preparation according to claims 16-18, obtained by the method according to claims 11-15.

22) An essentially pure preparation of differentiated stem cells, wherein the cells display the expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

23) The preparation according to claims 21, obtained by the method according to claims 11-15.

24) An essentially pure preparation of cells obtainable by the method according to claims 11-15.

25) Use of a preparation according to claims 16-20 or claim 23 for the manufacture of a medicament for the prevention or treatment of pathologies or diseases in the pancreas.

26) Use according to claim 25, in which the disease is diabetes.

27) Use according to claim 25 or 26, in which the disease is type 1 diabetes.

28)Use of a preparation according to claims 21-23 for the manufacture of a medicament for the treatment of pathologies or diseases in the nervous system.

29)Use according to claim 28, in which the disease can be chosen from the following diseases or disturbances; multiple schlerosis, spinal chord injury, encephalopathies, Parkinson's disease, Huntingdon's disease, stroke, traumatic brain injuries, hypoxia induced brain injuries, ischemia induced brain injuries, hypoglycemic brain injuries, degenerative disorders of the nervous system, brain tumors, or neuropathies in the peripheral nervous system.

30)Kit for performing the method according to claims 4-9, comprising at least two of the following components in separate compartments; hyaluronic acid, pronase, ES-cell medium, and human or mouse embryonic feeder cells

31)Kit according to claim 30 further comprising blastocysts with an intact zona pelludica or spontaneously hatched blastocysts.

32)Kit for performing the method according to claims 11-15, comprising at least two of the following components in separate compartments; mitomycin C, hES medium, EB-medium, ITSFn-medium, N2-medium, B27-media supplement, nicotinamide, and bFGF.

33)Kit according to claim 32, further comprising an essentially pure human embryonic stem cell line according to claims 1-3.

Abstract

The present invention concerns a method for the establishment of pluripotent human embryonic stem cells, the stem cells, differentiation of these cells into differentiated cells, the differentiated cells and the use of these differentiated cells in the preparation of medicaments. The undifferentiated pluripotent stem cells can be made to differentiate to a number of specialized cell types which can be utilized in the manufacture of medicaments for treating a number of pathologies of the pancreas, for example diabetes, or pathologies of the CNS, such as stroke, physical trauma, or Alzheimer's or Parkinson's diseases.

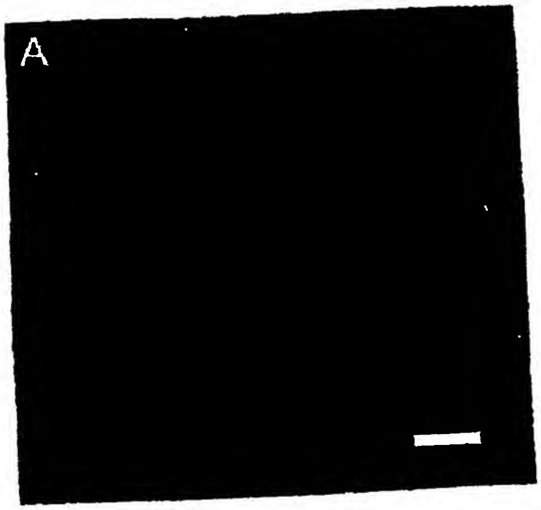


Figure 1A

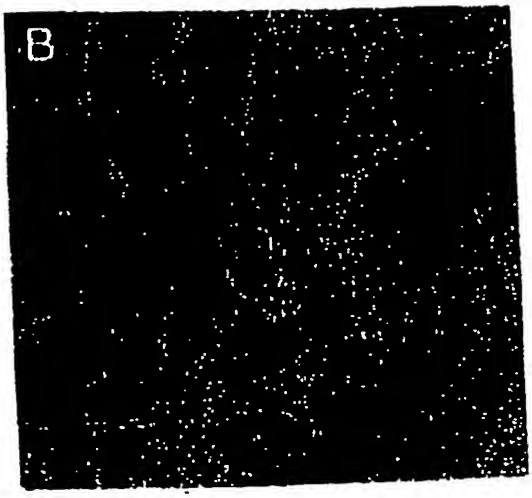


Figure 1B

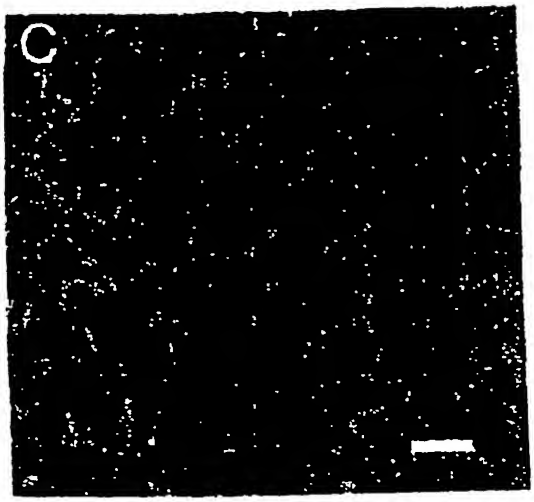


Figure 1C

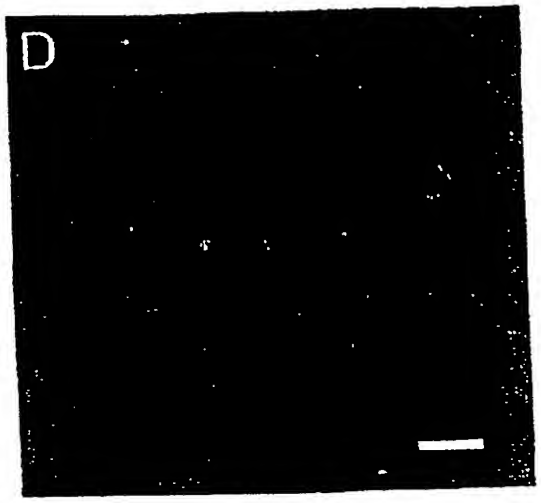


Figure 1D

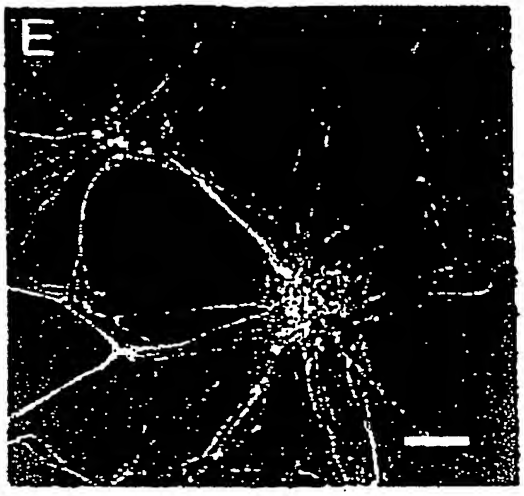


Figure 1E

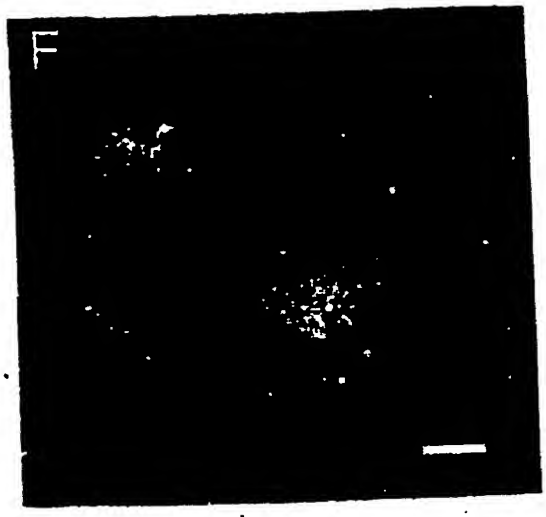


Figure 1F

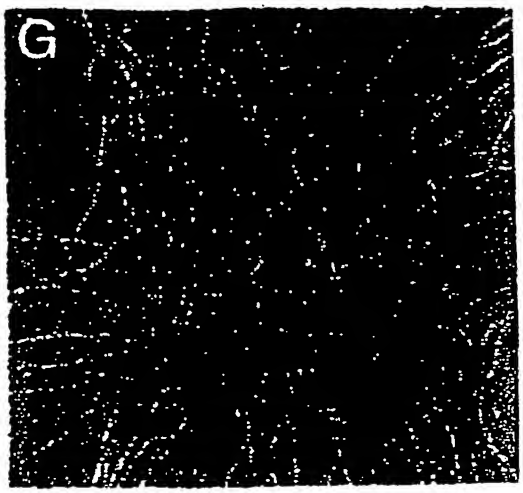


Figure 1G

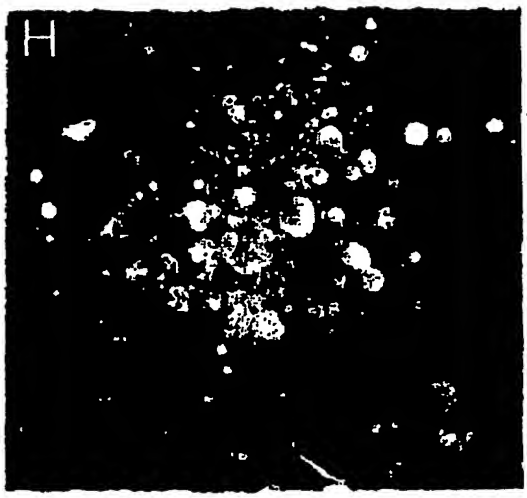


Figure 1H

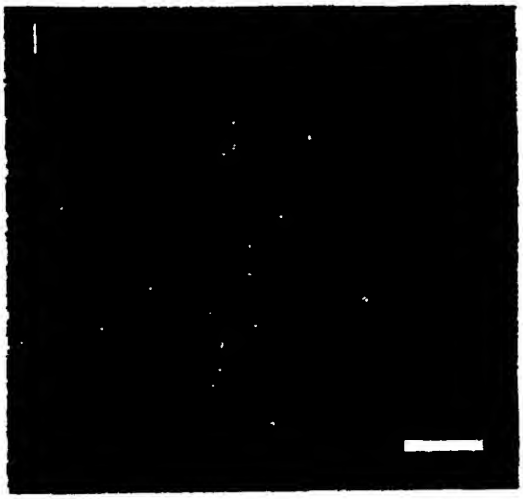


Figure 11

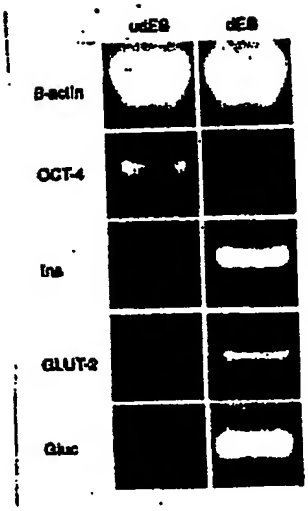


Figure 2

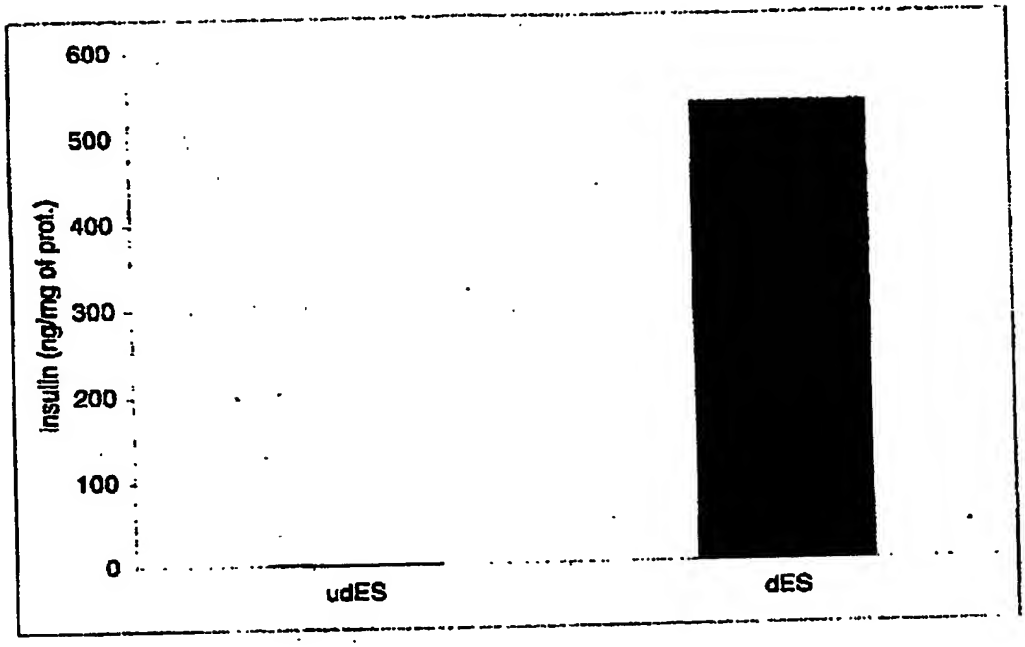


Figure 3

Figure 4A

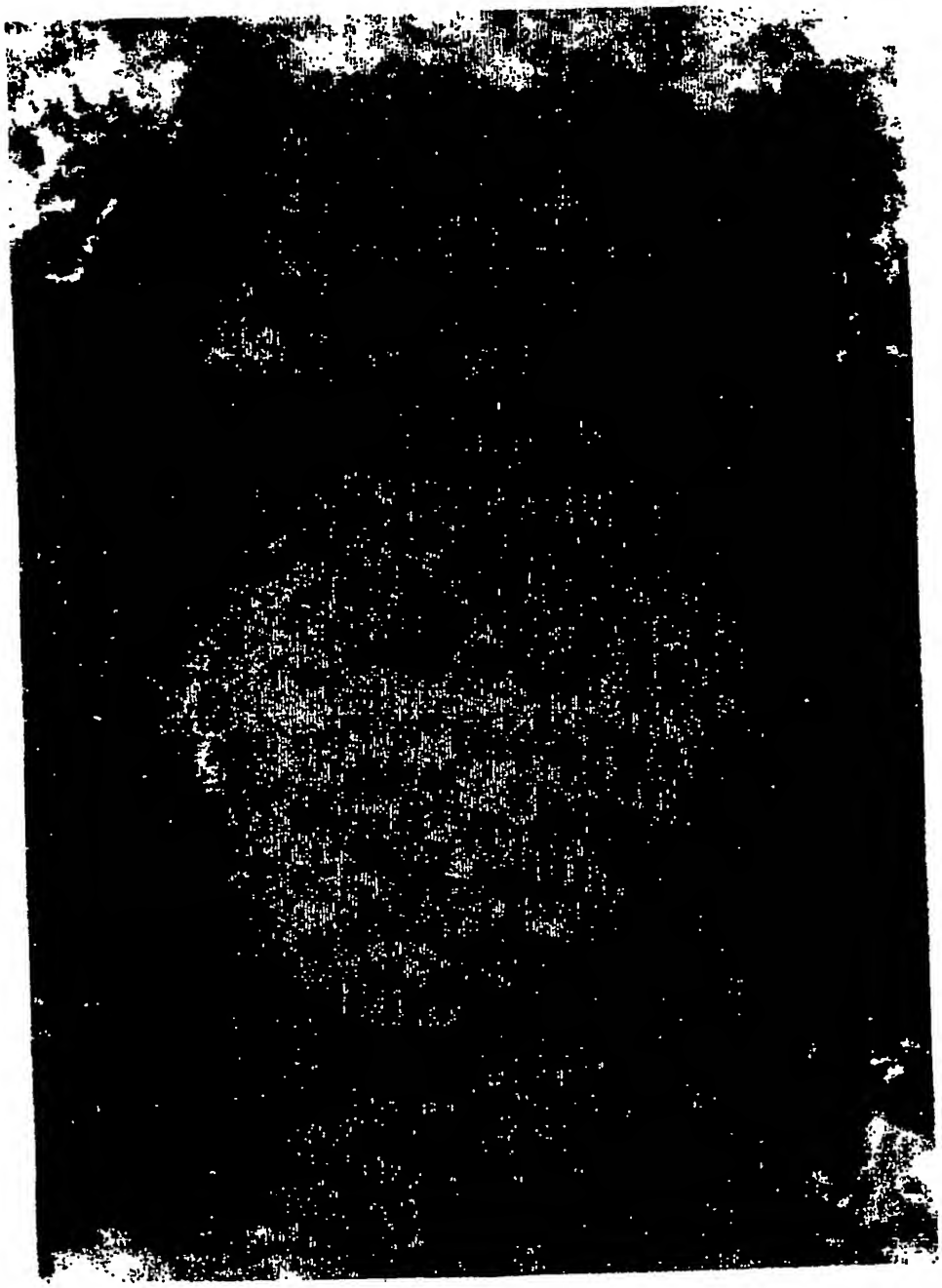


Figure 4B

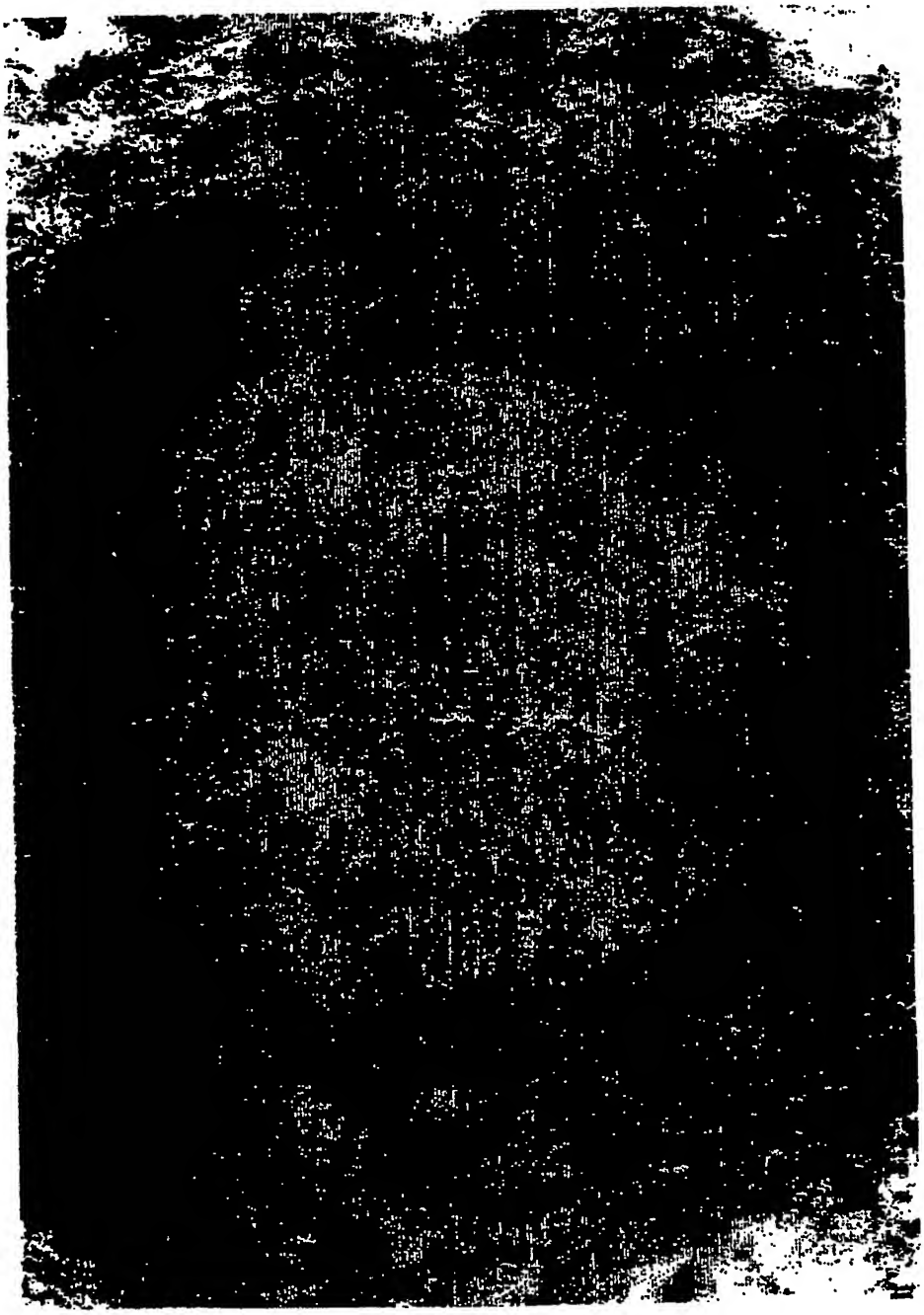


Figure 4C

Figure 4D

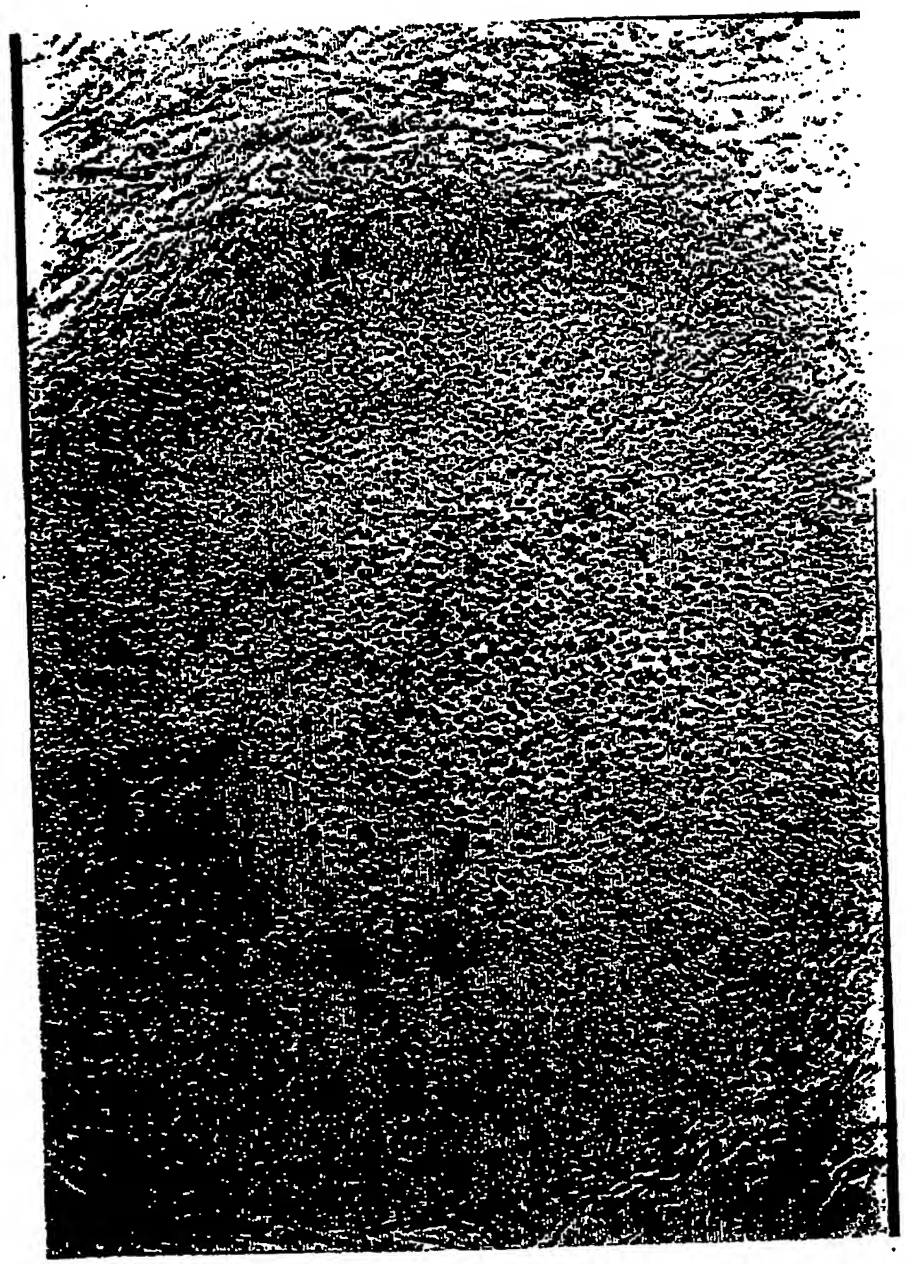


Figure 5A

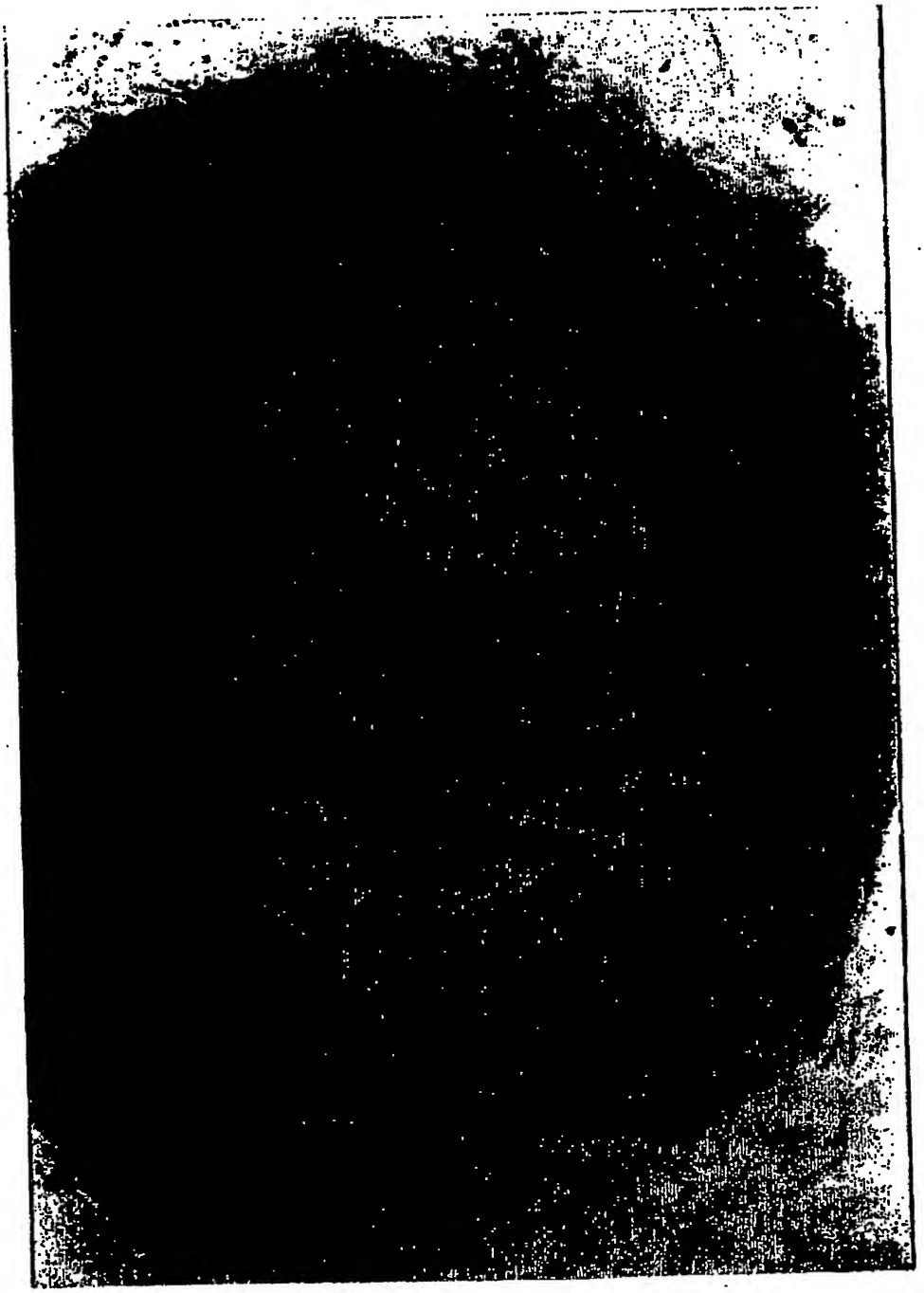


Figure 5B

Figure 5C

Figure 5D

Human embryonic cell line SA03

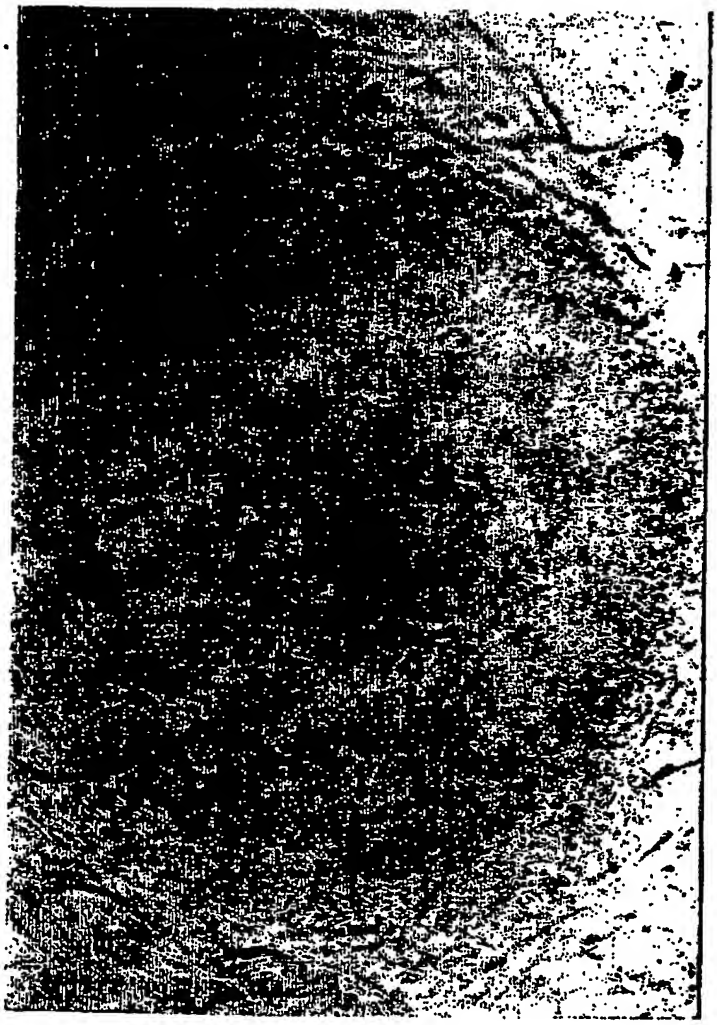
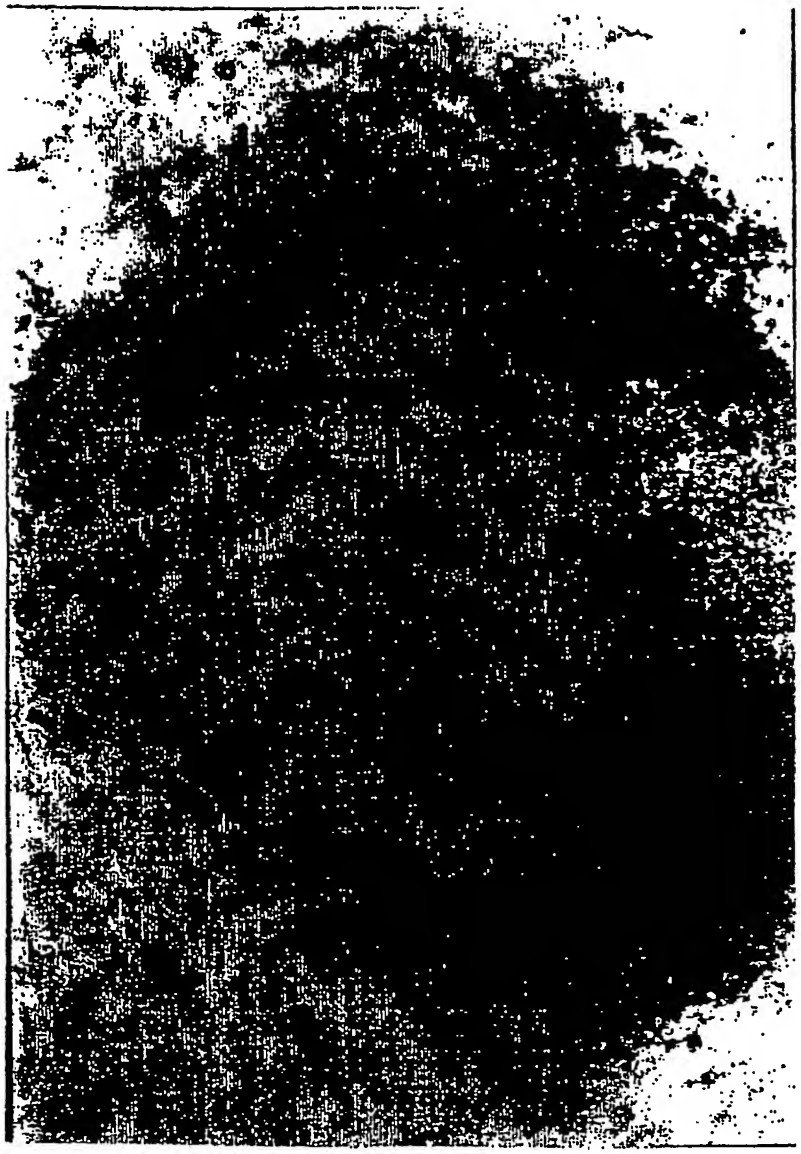


Figure 6A

Figure 6B



Human embryonic cell line SA03

Figure 6C

Human embryonic cell line SA03

[redacted]

Figure 6D